DR ANDREAS HAGENBO (Orcid ID : 0000-0002-4192-0511)

MR CARLES CASTAÑO (Orcid ID : 0000-0002-2403-7006)

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Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus* pinaster, *Pinus sylvestris* and *Quercus ilex* forests

Andreas Hagenbo[1,2,3,4], Yasmine Piñuela[2], Carles Castaño[5], Juan Martínez de Aragón[1], Sergio de-Miguel[1,2], Josu G. Alday[1,2], José Antonio Bonet[1,2]

[1]Joint Research Unit CTFC - AGROTECNIO, Av. Alcalde Rovira Roure 191, 25198 Lleida, Spain;
[2]Department of Crop and Forest Sciences, University of Lleida, Lleida E-251 98, Spain;
[3]School of Science and Technology, Örebro University, Örebro SE-701 82, Sweden;
[4] Norwegian Institute of Bioeconomy Research (NIBIO), Box 115, 1431 Ås, Norway;
[5] Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala SE-750 07, Sweden.

*Author for correspondence
Andreas Hagenbo
Tel: 0019 303408
Email: andreas.hagenbo@nibio.no

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ORCID numbers:
Andreas Hagenbo: 0000-0002-4192-0511
Yasmine Piñuela: 0000-0002-1150-7160
Carles Castaño: 0000-0002-2403-7006
Juan Martínez de Aragón: 0000-0001-5663-2080
Sergio de-Miguel: 0000-0002-9738-0657
Josu G. Alday: 0000-0001-7510-8655
José Antonio Bonet: 0000-0003-2209-9374

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Summary

- In forests, ectomycorrhizal mycelium is pivotal for driving soil carbon and nutrient cycles, but how ectomycorrhizal mycelial dynamics vary in ecosystems with drought periods is unknown. We quantified production and turnover of mycorrhizal mycelium in Mediterranean *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex* forests and related the estimates to standardized precipitation index (SPI), to study how mycelial dynamics relates to tree species and drought-moisture conditions.

- Production and turnover of mycelium was estimated between July-February, by quantifying the fungal biomass (ergosterol) in ingrowth mesh bags and using statistical modelling. SPI for time scales of 1 to 3 months, was calculated from precipitation records and precipitation data over the study period.

- Forests dominated by *Pinus* trees displayed higher biomass but were seasonally more variable, as opposed to *Q. ilex* forests where the mycelial biomass remained lower and stable over the season. Production and turnover respectively varied between 1.4-5.9 kg ha^{-1} day^{-1} and 7.2-9.9 times $year^{-1}$ over the different forest types and were positively correlated with 2- and 3-month SPI over the study period.

- Our results demonstrate that mycorrhizal mycelial biomass vary with season and tree species and we speculate that production and turnover are related to physiology and plant-host performance during drought.

1 Introduction

Soil fungi play a pivotal role in driving processes regulating nutrient and carbon cycling in forest ecosystems (Baldrian, 2016), which feedback on plant productivity as well as on ecosystem responses to climate and environmental changes (Mohan *et al.*, 2014). Symbiotic root-associated mycorrhizal fungi are one of the most important functional groups of the soil microbiome in regard to plant growth

and cycling of soil carbon (C) and growth-limiting nutrients, in particular nitrogen (N) and phosphorous (P). The mycelia of mycorrhizal fungi extend into the soil to forage for growth-limiting soil nutrients, which are transferred to the host plant in exchange for photosynthetically fixed carbohydrates. In forest ecosystems, the partitioning of C to belowground vary across conditions (Litton *et al.*, 2007), but usually 50-60% the photosynthetic C is allocated belowground (Gill & Finzi, 2016), and about half of this (25% of the C budget) is thought to be received by the mycorrhizal fungi (Simard *et al.*, 2003; Leake *et al.*, 2004). Although the majority of allocated C is likely released via respiration (Hagenbo *et al.*, 2019), a significant fraction is directed to the production of mycelium, which often exceeds several hundred kilograms per hectare and year (Ekblad *et al.*, 2013). The mycelial biomass has a strong feedback effect on soil C cycling and plant productivity (Orwin *et al.*, 2011; Baskaran *et al.*, 2017), and its size is simultaneously regulated by the rate of production (growth) and the rate of turnover (death and autolysis) (Rousk & Bååth, 2007; Ekblad *et al.*, 2016). While production and turnover of mycelium constitutes an important pathway of C into the soil, the factors controlling mycelial dynamics remain unclear. Mycorrhizal mycelial production is considered to be coupled with allocation of C from the plant host (Wallander, 1995; Ekblad *et al*., 2013), and plant C allocation is thought to decrease as nutrient availability increases, as the C allocation cost for trees begins to outweigh the obtained benefit (Treseder & Allen, 2002). Drought conditions constrain photosynthesis and thus plant growth. Under moderate drought conditions, host plant's C investment into the mycorrhizal association appears to increase (Shi *et al.*, 2002), but decreases under severe water stress (Staddon *et al.*, 2002; Swaty *et al.*, 2004). However, the extent to which dry conditions affect mycorrhizal mycelial dynamics is not well known, which severely hampers predictions of forests ecosystems responses to climate change (Deckmyn *et al.*, 2014).

Mediterranean forests are often constrained by limited water availability, and ecosystem responses to drought vary with tree species dominance (Pasho *et al.*, 2011; Camarero *et al.*, 2015). Rooting depth of trees determines their capacity to access deep soil layers which usually hold water reserves during the dry season (Schulze *et al.*, 1996). *Quercus ilex* L. stands among the deepest rooted tree species in Mediterranean ecosystems (Joffre *et al.,* 1999), and under drought conditions, *Q. ilex* may keep stomata open while maintaining a low stomatal conductance to support photosynthesis and root growth to deep water reservoirs (Manes *et al.*, 2006). *Pinus sylvestris* L. typically occurs at rather

high altitudes in Mediterranean areas, where summer drought is less severe, whereas *Pinus pinaster* Ait. thrives in mid-altitude Mediterranean areas characterized by hot and dry summers and less frequent frosts during winter. Mediterranean *P. pinaster* grows roots faster and develops larger root systems than *P. sylvestris*, contributing to its greater capacity to colonize drier Mediterranean sites (Andivia *et al.*, 2019). Indeed, forests dominated by *P. sylvestris* have suffered frequent episodes of drought-induced dieback in its southernmost peripheral population (Galiano *et al.*, 2010), whereas Mediterranean *P. pinaster* forests at the southern distribution limit have demonstrated a high plasticity in their growth responses to drought (Caminero *et al.*, 2018).

Several different tree species coexist along Mediterranean elevation gradients, characterized by changing climatic conditions and vegetation types (Tapias *et al.*, 2004), and recent studies have provided evidence of climate-induced shifts in fungal sporocarp community structure and dynamics (Andrew *et al.*, 2016; Alday *et al.*, 2017). Most Mediterranean tree species are able to reduce their growth and transpiration to avoid water stress during dry periods (Baldocchi *et al.*, 2010), and different responses to drought may affect belowground C allocation (Litton *et al.,* 2007), with feedbacks on mycorrhizal-mediated processes and mycelial dynamics. However, the extent to which mycorrhizal mycelial dynamics vary with tree species in Mediterranean climates remains unknown.

Drought is complex and vary in regard to duration, magnitude, severity and frequency. The Standardized Precipitation Index (SPI) is widely used to identify and characterize precipitation deficits for multiple timescales (McKee *et al.,* 1993). The SPI values indicate the standard deviations by which an observation deviates from the long-term mean so that values above zero indicate moist conditions and negative values indicates dry conditions. SPI calculated for 1- to 3-month generally represent availability of water of short-term reservoirs, such as water stored within soil pores, and relates to plant water stress (WMO, 2012; Halwatura *et al.*, 2017).

In the present study, we assembled a Mediterranean elevation gradient to test how mycorrhizal mycelial production and turnover rates vary with 1- to 3-month SPI in forest ecosystems dominated either by *P. pinaster*, *P. sylvestris* or *Q. ilex* trees, in accordance to their different drought responses and water-use characteristics. In the study area, *P. sylvestris* is near its southern distribution limit, as opposed to *P. pinaster* and *Q. ilex* which are widely distributed in the region and display a high phenotypic plasticity in responses to drought (Gratani *et al.*, 2003; Pasho *et al.*, 2011; Caminero *et al.*,

2018). Production and turnover rates were established from fungal biomass estimates, derived from mycelial ingrowth mesh bags, incubated over different and overlapping incubation periods (Ekblad *et al*., 2016). Estimates of mycelial production and turnover were also regressed against sporocarp production, altitude and stand basal area to explore potentially significant relationships (Bonet *et al*., 2010). Additionally, variation in mycorrhizal mycelial biomass ingrowth was investigated over the different forest types and over July-February, to assess how biomass dynamics of mycorrhizal mycelium vary over late-summer to early-spring.

We hypothesized that (*i*) *Q. ilex* dominated forests would display a lower seasonality in mycorrhizal mycelial biomass as well as lower production and turnover rates compared to forests dominated by *P. pinaster* and *P. sylvestris*. This hypothesis was drawn from *Q. ilex* having a deep root system to accommodate water stress (Joffre *et al.,* 1999), and high stomatal sensitivity to drought (Mediavilla & Escudero, 2003), and observations of lower sporocarp production in *Q. ilex* compared to *Pinus* stands in the study area. We thus assumed that the factors regulating sporocarp production are similar to factors regulating mycorrhizal mycelial dynamics (Castaño *et al.*, 2017) and that *Q. ilex* forest have a lower, but more stable belowground C allocation following the summer drought.

We also hypothesized that (*ii*) production and turnover of mycelium would increase with 1- to 3-month SPI, as an effect of improved water conditions. This hypothesis was based on previous findings of an enhanced mycorrhizal biomass production following improved water availability (Sims *et al.*, 2007) and that tree growth is strongly controlled by precipitation (Pasho *et al.*, 2011; Shestakova *et al.*, 2017; Collado *et al.*, 2018, 2019). We thus assumed that forest stands subjected to less severe drought conditions perform better in terms of growth and belowground C allocation.

2 Materials and methods

2.1 Study sites

The study was conducted in eleven Mediterranean forest stands, dominated by even-aged trees of either *Pinus pinaster* (Aiton), or *Pinus sylvestris* (L.) or *Quercus ilex* (L.), and located between 530 to 1013 m.a.s.l. Forests dominated by *Pinus pinaster* and *Pinus sylvestris* were each represented by four forest plots and forest dominated by *Q. ilex* trees was represented by three plots. All plots were

located in the Natural Protected Area of Poblet, northeastern Spain (41°21' 6.4728" E, 1°2' 25.7496" N), which is an experimental area used in previous research, to quantify sporocarps production and soil fungal diversity in Mediterranean forests (Bonet *et al.,* 2012; Castaño *et al.*, 2018a,b; Collado *et al.,* 2018). The soils are classified as a calcic cambisol (FAO, 1998) characterized by siliceous minerals with sandy loam textures, with pH ranging from 6.1 to 6.6. Understory vegetation was sparse and mainly composed by *Erica arborea* (L.), *Arbutus unedo* (L.) and *Calluna vulgaris* ((L.) Hull). Mean annual temperature and total annual precipitation ranged from 10.8-14.5°C and from 514-658 mm, respectively, with summer droughts usually occurring between July and September. See Table S1 in Supporting Information for further details.

2.2 Experimental design, mesh bags and sampling of sporocarps

Mycelial ingrowth mesh bags (100 × 20 mm) made from a 50 µm nylon mesh (Sintab Produkt AB, Malmö, Sweden), were used to sample mycorrhizal mycelium from the soil. The mesh bags were filled with 40g of acid-washed silica sand (0.36-2.0mm, 99.6% SiO_2, Brico Depôt, Lleida, Spain) to allow standardized comparison over the different forest types and plots, and because sand-filled mesh bags have repeatedly demonstrated to select for mycorrhizal fungal ingrowth over a wide different settings (Wallander *et al.*, 2001, 2010; Parrent & Vilgalys, 2007; Kjøller *et al.*, 2012; Hagenbo *et al.*, 2018). Sand-filled mesh bags discriminate against saprotrophic fungal ingrowth as mycorrhizal fungi are not energetically dependent on degradation of organic C in the soil, thus are able to colonize the bags more easily compared to fungal saprotrophs. Mycorrhizal mycelia dynamics can be assessed by incubating mesh bags over different and overlapping incubation periods (Wallander *et al.,* 2013; Ekblad *et al.,* 2016). In this study, mesh bags were incubated according to the incubation scheme in Figure 1, which was replicated in each of the eleven forest plots and involved six different sets of mesh bags (a-f), each set consisting of five replicated bags. Thus, a total of 330 bags were used. The mesh bags were allocated within a 10 × 10 m area located in the middle of each stand, and were inserted to 7-cm depth into the soil at an angle of 45° by making a hole using a garden trowel with a 4-cm wide scoop-shaped metal blade. Incubation time of mesh bags ranged between 49 and 121 days and upon harvest of mesh bags sets (*i.e*. at the beginning of September, and at the end of October, December and February), new bags were installed into the same hole as the preceding bags, to

minimize effects of soil disturbance. No additional mesh bags were installed at the final harvest in February. After each harvest, the bags were stored in the dark and transferred to -20°C storage within few hours. Frozen mesh bags were freeze dried, and the contents of five replicated bags, representing the same plot and incubation period, were pooled and ground using mortar and pestle.

Moreover, each week during September-December of the study period, all epigeous sporocarps were harvested from each plot. Sporocarps were identified to genus or species level based on morphological features, and classified as saprotrophic or ectomycorrhizal, according to Agerer (2006) and Tedersoo & Smith (2013). The dry biomass of the sporocarps was determined after several days of drying, and monthly production of sporocarps was determined from the total dry weights (DW). Production of sporocarps prior September was negligible.

2.3 Analyses of free ergosterol and estimation of fungal biomass

From pooled mesh bags samples, representing the same plot and incubation period, fungal biomass was quantified by analyzing the fungal-specific biomass marker ergosterol. Ergosterol was extracted as described by Nylund and Wallander (1992) but with the modification that pure methanol was used instead of 10% KOH in methanol (Wallander *et al.*, 2010), to only extract free ergosterol to get a better indication of freshly produced mycelium (Wallander *et al.*, 2013). Free ergosterol is present mainly in the plasma membrane (Bloch, 1983) where it contributes to functioning of its bound proteins, responsible for nutrient transport and chitin synthesis (Bloch, 1983). Free ergosterol has been suggested to be a better proxy for living fungi compared to total ergosterol (Yuan *et al.*, 2008), which also includes esterified (bound) forms of ergosterol. Three to six technical replicates were used for each sample and all extracts were filtered through a Teflon 0.22 µm syringe filter (Simplepure, Membrane Solutions, Auburn, WA, USA). Following extraction, ergosterol was chromatographically quantified using a UPLC system (ACQUITY UPLC, Waters, Milford, CT, USA), consisting of a triple quadrupole mass spectrometer (Xevo TQ-S; Waters, Milford, CT, USA) equipped with an atmospheric pressure chemical ionization source (Sun *et al.*, 2005). Chromatographic separation was done using CORTECS C_{18} analytical column (1.6 µm, 2.1 × 100 mm), methanol was used a mobile phase, and the analyses were conducted using multiple reaction monitoring mode.

2.4 *Climate data*

Monthly precipitation data was obtained from 2008-2019 for each of the eleven plots using the DAYMET methodology (Thornton *et al.*, 2000), as implemented in the R package 'meteoland' version 0.5.9 (De Cáceres *et al.*, 2018). In short, precipitation was estimated for each plot by averaging the values of several local meteorological stations, applying weighting factors that depended on the station's geographical proximity to the target plot and correcting for elevation differences between plot and stations. From monthly precipitation data obtained from 2008-2019, 1-, 2- and 3-monthly standardized precipitation index (SPI) was calculated for all sites and months of the study period (July 2018 – February 2019, using the 'precintcon' R package (Povoa & Nery, 2016). The SPI is widely used to identify and characterize drought (Anshuka *et al.*, 2019), and is bases on precipitation records that are computed on different time scales (McKee et al.1993). The time scales of SPI (usually 1 to 42 months) reflect the availability of different water sources, *e.g.* soil moisture, stream flows and ground water reservoirs, depending on the length of the calculated period (McKee et al.1993; Halwatura *et al.*, 2017). Ideally, 20-30 years of monthly precipitation values should be used to obtain robust SPI values (WMO, 2012). In the present study this was not possible and therefore the monthly values were aggregated over the entire study period (July - February) to represent an average index of the moisture conditions. Additionally, the error related the short precipitation record (11 years) was assumed to be equal across sites, thus still enable relative comparisons, and only short time-scales (1 to 3 months) SPI was considered, which are less sensitive to long precipitation records (Wu *et al.,* 2005).

2.5 Calculations

Fungal biomass was calculated from the ergosterol measurements using a conversion factor of 3 μg ergosterol/mg fungal dry matter (Salmanowicz & Nylund, 1988), and a correction factor (1/0.62) was applied to compensate for un-extracted mycelial ergosterol (Montgomery *et al.*, 2000).

Production and turnover of mycorrhizal mycelium was estimated for each plot by fitting an exponential decay model (Eqn 1) to ergosterol-derived fungal biomass estimates (Ekblad *et al.*, 2016), representing the same site but different incubation periods and period lengths (a-f in Fig. 1). The model describes the temporal change in mycelial biomass ingrowth ($B(t)$) as a function of incubation

time (t) of the mesh bags, production (p) in units of biomass per unit of time, and turnover (μ), which represents the replacement rate of biomass per unit of time, caused by death and autolysis.

$$B(t) = \frac{p}{\mu}(1 - e^{-\mu t}) \qquad \text{Eqn 1}$$

In the study area, variation in standing fungal biomass is driven by the abundance of mycorrhizal fungi, which dominates the soil fungal communities (Castano *et al.,* 2018b). By using sand-filled ingrowth mesh bags, majority of the biomass is assumed to be of mycorrhizal origin, as demonstrated by community profiling and ^{13}C isotope analyses (Wallander *et al.*, 2001, 2010; Parrent & Vilgalys, 2007; Kjøller *et al.*, 2012; Hagenbo *et al.*, 2018). Additionally, the model assumes stable production and turnover rates over time and violation of this assumption adds uncertainty to the estimates (Ekblad *et al.,* 2016). To enable assessments of the reliability of the estimates, as well as account for scatter in the data, caused by variation in production and turnover over time, the estimation of production and turnover was obtained by parametric bootstrapping of Eqn 1. In short, biomass data was generated around a normal distribution, from the mean and standard deviation of the technical replicates, and a chain of 500 runs of the model was used to repeatedly fit the model to the generated biomass estimates using least squares fitting. Production and turnover was estimated from the mode value of the parametric estimates, derived from a kernel density distribution, as the probability distributions of the parameters might be skewed and, thus, the choice of the mode offers a more robust estimate than the mean (Ekblad *et al.*, 2016). Model fitting was done using the "minpack.lm" package (Elzhov *et al.*, 2016) for nonlinear least squares fitting in R, version 3.5.2 (R Core Team, 2017).

2.6 Statistical analysis

Relationships between parametric estimates of mycelial production and turnover and the average monthly SPI, sporocarp production, altitude and stand basal area were evaluated for statistical significance using linear regression. Linear regression was also fitted between the empirical mycelial biomass estimates and the predicted mycelial biomass obtained from Eqn 1 parameterized by the production- and turnover estimates. Multiple linear regressions were performed to evaluate the error

between the empirical biomass estimates and the predicted mycelial biomass, and to test the effects of sampling time (seasonality), forest type (*P. pinaster*, *P. sylvestris*, *Q. ilex*) and incubation time of the mesh bags on the mycelial biomass estimates. Locally estimated scatterplot smoothing was applied to the biomass estimates to visualize the seasonality (July-February) in mycelial biomass. All statistical analyses were performed in R version 3.5.2 (R Core Team, 2017).

3 Results

3.1 Variation in mycelial biomass ingrowth over the season and different forest types

A multiple linear regression analysis (adjusted $R^2 = 0.27$) highlighted that variation in mycelial biomass ingrowth was significantly related to forest type, *i.e. P. pinaster*, *P. sylvestris* or *Q. ilex* dominated forest, and harvest time of the mesh bags (Table 1). Mesh bags incubated in forests dominated by *Q. ilex* displayed a smaller biomass compared to *P. pinaster* forests ($P = 0.001$). Furthermore, mesh bags harvested in December also contained a significantly smaller biomass compared to mesh bags sampled in October ($P = 0.038$) and February ($P = 0.023$; Table 1). Over the season, mycorrhizal mycelial biomass in stands dominated by *P. pinaster* and *P. sylvestris* followed similar trends and displayed a bimodal seasonality with two seasonal peaks; the first one occurring in October-November, after the summer drought, and another occurring at the end of February (Fig. 2d). Conversely, mycelial biomass in *Q. ilex* forests showed weak trends of seasonality and remained relatively constant over the season (Fig. 2d). Mycelial biomass was not related to incubation duration of the mesh bags (Table 1), so that mesh bags incubated for two- and four mounts contained similar amounts of biomass (Fig. 2 a-c). Scaled up over a hectare, fungal biomass in mesh bags incubated over two and four months represented on average, 222, 142, and 62 kg ha^{-1} for *P. pinaster*, *P. sylvestris* and *Q. ilex* forests, respectively (Fig. 2a-c).

3.2 Variation in sporocarp biomass over the season and different forest types

The mushroom fruiting season in year 2018 began at the end of September, and production of non-mycorrhizal (*i.e.* saprotrophic) sporocarps reached a peak in October, with a total average production of 2.3 DW kg ha^{-1} across all forest types, whereas production of mycorrhizal sporocarps reached a

peak in November, with a total average production of 9.1 DW kg ha^{-1} across the forest types (Fig. 3). In November, total (mycorrhizal + saprotrophic) sporocarp production was 10.4, 20.0 and 2.9 DW kg ha^{-1} in the *P. pinaster*, *P. sylvestris* and *Q. ilex* forests (Fig. 3), respectively, representing 78, 86 and 68% of the total sporocarp production during that month. In December, 95-99% of the sporocarp production was represented by ectomycorrhizal fungi (Fig. 3). Across the season (September–December) and all forest types, total production of mycorrhizal and saprotrophic sporocarps was 143 and 50.8 kg ha^{-1}, respectively. The most predominant ectomycorrhizal sporocarps were represented by species within the genus *Lactarius* and *Tricholoma*, whereas species within the genus *Macrolepiota* and *Mycena* dominated the production of saprotrophic sporocarps. See table S2 for a taxonomic break down of fungal sporocarps.

3.3 Production and turnover rates of mycorrhizal mycelial biomass

Mode values of the parametric estimates of production ranged between 2.2-11.1; 1.7-7.4 and 1.1-12.8 kg ha^{-1} day^{-1}, for forests dominated by *P. pinaster*, *P. sylvestris* and *Q. ilex*, respectively (Fig. 4, Table S3). Median production for the respective forests stands was 5.9, 5.1 and 1.4 kg ha^{-1} day^{-1}, and 5.4 kg ha^{-1} day^{-1} for all the forest types combined (Fig. 5a). Conversely, mode values of the parametric estimates of turnover ranged between -3.9-17.8; 5.5-11.3 and 3.3-66.2 times $year^{-1}$ for *P. pinaster*, *P. sylvestris* and *Q. ilex* forests (Fig. 4), corresponding to a median turnover of 9.9, 8.6 and 6.6 times $year^{-1}$ and a mycelial longevity of 37, 42 and 55 days for the respective forest types (Fig. 5b). There was no significant difference in production and turnover between the forest types, and the median turnover for all forest types combined was 6.9 times $year^{-1}$, corresponding to a mycelial longevity of 53 days (Fig. 5b).

Linear regressions resulted in positive correlations ($P < 0.05$) between 2 and 3-month SPI and estimates of mycelial production and turnover (Fig. 6; Fig S1). Additionally, 1-month SPI was significantly related to turnover ($R^2 = 0.45$; $P = 0.023$; Fig S1c), and at $\alpha = 0.1$, mycelial production was significantly related to 1-month SPI ($R^2 = 0.36$, $P = 0.051$; Fig. S1a) and to the sporocarp production of December ($R^2 = 0.28$; $P = 0.093$). Production and turnover were not significantly related to altitude nor stand basal area.

3.4 Evaluation of the production and turnover estimates

Using the parametric production and turnover estimates (Fig. 3) to parametrize a growth model (Eqn 1) quantifying the observed mycelial dynamics, the model predicted 50% of the observed variation in mycorrhizal mycelial biomass ($P < 0.001$; Fig. 7a). Predictability varied over the season (Table S4), and partitioning of the data according to harvest time points (September, October, December and February), yielded models with R^2 values ranging from 0.30-0.78 (Fig. 7b-e). Predictability of biomass was lowest for September ($R^2 = 0.30$; $P = 0.081$; Fig. 7b) and highest for October ($R^2 = 0.78$; $P < 0.001$; Fig. 7c). Furthermore, the model (Fig. 7a) tended to over- and underestimate the biomass in mesh bags incubated over two- and four months, respectively (Table S4).

4. Discussion

4.1 Seasonality in biomass varied with tree species and production and turnover rates increased with improved moisture conditions

In the present study we investigated mycorrhizal mycelial biomass dynamics over late-summer to early-spring and quantified production and turnover of mycorrhizal mycelium in Mediterranean *P. pinaster, P. sylvestris* and *Q. ilex* forest stands. In agreement to our first hypothesis, the mycelial biomass of *Q. ilex* remained relatively constant over the study period, as opposed to the mycelial biomass in *Pinus* dominated forests, which declined at early-autumn and early-winter. In agreement to our second hypothesis, production and turnover of mycorrhizal mycelium increased with 2- and 3-month SPI, and with 1-month SPI at $\alpha = 0.1$ ($P = 0.051$), generally representing short-term moisture conditions, *e.g.* soil moisture and precipitation (WMO, 2012). The findings of this study highlight that mycelial dynamics of mycorrhizal fungi in Mediterranean forests are likely constrained by lack of water (Castaño *et al.,* 2017; 2018b). Water limitations may directly restrict mycorrhizal growth by immediate water stress, or indirectly via reduced host tree performance (Fernandez *et al.*, 2017), reducing allocation of C to belowground and, thus, limiting the mycorrhizal C availability. Furthermore, water is required for functioning of hydrolytic enzymes of mycorrhizal fungi, and restricted water access which is likely to have consequences on nutrient availability by reducing enzyme's capacity to degrade soil organic matter (Sardans & Peñuelas, 2013). Under increased

drought severity following climate change (Nogués-Bravo *et al.*, 2008), it is possible that mycorrhizal mycelial dynamics may shift towards slower growth and turnover in forest types with poor drought-adaptations, which may negatively affect forest growth and soil nutrient cycling (Orwin *et al.*, 2011). Slow growth and turnover have been observed in old boreal forests (Hagenbo *et al.,* 2017; 2018), which are characterized by slow N cycling and less labile nutrient pools compared to young forests (Bauhus *et al.*, 1998). As a result of deep water uptake, tree species with deep roots, are generally less adversely affected by drought compared to species with more shallow roots (Schulze *et al.*, 1996). Better access to deep water reservoirs in *Q. ilex* stands could result in more stable conditions and contributing to a lower seasonality in mycelial biomass. For example, access to groundwater can favor water uptake of trees by hydraulic lift, which can eventually be transferred to its associated symbionts (Querejeta *et al.*, 2003, 2007; Lilleskov *et al.*, 2009). Opposed to *P. sylvestris* and *P. pinaster*, *Q. ilex* is a slow growing trees species (Crescente *et al.*, 2002), and during summer drought displays a low net CO_2 assimilation together with a high stomatal control reducing transpiration (Mediavilla & Escudero, 2003), and potentially this could contribute to the observed low mycelial biomass and lack of seasonality. Conversely, the observed seasonal change in mycelial biomass ingrowth of *Pinus* spp. stands is similar to other studies reporting decreases in ectomycorrhizal abundance following drought (Iotti *et al.*, 2014; Queralt *et al.*, 2017; Castaño *et al.*, 2017).

Trees affected by drought may limit growth and increase allocation of C to belowground root system and root-associated mycorrhizal fungi to retain sufficient water uptake (Ibrahim *et al.*, 1998; Aaltonen *et al.*, 2017). However, drought may also induce stomatal closure and constrain the photosynthetic capacity of trees, and thus limit the allocation of C to belowground roots and associated microorganisms (Fuchslueger *et al.*, 2014; Hasibeder *et al.*, 2015). Although the responses of belowground C allocation to drought remains unclear, seasonal variation in belowground C allocation could contribute to the observed seasonality in biomass of *Pinus* spp. stands, as even mild droughts have been shown to decrease mycorrhizal colonization in boreal and temperate forests (Lehto & Zwiazek, 2011). Potentially, drought may also shift fungal community composition towards an increased abundance of drought-resistant species with a lower mycelial biomass and with specific functional adaptations against water stress (Smith *et al.*, 2007; Gordon & Gehring, 2011). The mycelial architecture of mycorrhizal fungal species has been used to describe different species traits

and mycorrhizal growth forms (Agerer, 2001). Mycorrhizal species forming extensive mycelial networks (*e.g.* medium-, fringe-, and long-distance exploration types) may imply a higher C demand on the host, as more energy would be required to support the maintenance of a large biomass (Rygiewicz & Andersen, 1994), while species forming small mycelial networks (*e.g.* contact – and short-distance exploration types) have been demonstrated to increase in abundance under dry conditions (Fernandez *et al.*, 2017; Castaño *et al.*, 2018b). The extent to which belowground C allocation changes with drought likely relates to belowground C demands which likely vary between forests types because of differences in mycorrhizal community compositions. Given the smaller mycelial biomass (60 kg ha^{-1}) in mesh bags incubated in *Q. ilex* forests (compared to *Pinus* forests; 182 kg ha^{-1}) it seems likely that 'low-biomass' mycorrhizal fungal species (contact or short-distance exploration types) may be more abundant in such forest ecosystems (Agerer, 2001). A smaller biomass could impose a lower C cost for the host plant (Godbold *et al.*, 1997), and such a low belowground C demand, together with a greater drought tolerance, could contribute to a lower mycelial seasonality, as in *Q. ilex* forests. However, it is uncertain if a large biomass indicates a high C demand as the rate of growth could be the primary factor determining the C demand of mycorrhizal fungi (Koide *et al.,* 2014). Nevertheless, given the overall slow growth of *Q. ilex* (Crescente *et al.*, 2002), and the observed low mycelial biomass and variability it seems likely that the mycorrhizal community of *Q. ilex* stands are tailored to low C supplies.

4.2 Rapid production and turnover of mycorrhizal mycelium in Mediterranean forests

We hypothesized that *Q. ilex* forests would have a lower production and turnover of mycorrhizal mycelial biomass compared to *P. pinaster* and *P. sylvestris* dominated stands. This hypothesis was rejected as the differences in mycelial production and turnover between forest types were not significant. Across the different forest types, the production estimates ranged from 1.4 to 5.9 kg ha^{-1} day^{-1}, and the turnover estimates ranged from to 7.2 to 9.9 times $year^{-1}$, corresponding to a mycelial longevity of 37 to 51 days. Most previous research on mycorrhizal mycelial biomass in soils has been conducted in boreal and temperate ecosystems (Ekblad *et al.*, 2013). However, Castaño *et al.,* (2017) investigated mycelial dynamics of the ectomycorrhizal fungus *Lactarius vinosus* in *P. pinaster* forests, and found that production was on average, 2.2 kg mycelium ha^{-1} day^{-1} over a year, and that

turnover was 7.0 times year^{-1}, corresponding to mean longevity of 51 days. In comparison, we estimated that the mycelial production and turnover, respectively, was 5.9 kg ha^{-1} day^{-1} and 9.9 times year^{-1} between September-February in *P. pinaster* forests. Compared to Castaño *et al.,* (2017), the generally higher mycelial turnover observed in *P. pinaster* forests of the current study could be related to the fact that our study was conducted during several periods of mycelial decline, evidently from the observed seasonality in mycorrhizal mycelial biomass ingrowth. Furthermore, our higher production estimates are likely the result of sampling the majority of the mycorrhizal fungal community, rather than the biomass of *L. vinosus* alone, which is frequently occurring in the form of sporocarps in *P. pinaster* forests of the study area (Bonet *et al.,* 2012; Collado *et al.,* 2018).

Over a chronosequence of hemiboreal *P. sylvestris* forest stands aged 12 to 158 years old, production and turnover rates ranged from 0.5 to 1.2 kg ha^{-1} day^{-1} and <1 to 7 times year^{-1}, respectively (Hagenbo *et al.*, 2017, 2018). Furthermore, in control plots of a 25-year-old *Pinus palustris* forest, Hendricks *et al.,* (2016) found production and turnover to be 0.8 kg ha^{-1} day^{-1} and 10 times yr^{-1}, respectively, and in control plots of a 27-year-old *Pinus taeda* forest Ekblad *et al.,* (2016) reported production and turnover to be 1.3 kg ha^{-1} day^{-1} and 13 times year^{-1}, respectively. Our turnover estimates are similar to the ones reported by Hendricks *et al.,* (2016) and Ekblad *et al.,* (2016), but higher than the estimates reported in Hagenbo *et al.,* (2018). Growing season length of boreal ecosystem typically extents over 180 days, and the higher turnover of the present study is likely an effect of different growing season lengths between boreal and Mediterranean ecosystems. While dividing our turnover estimates by (365/180), to compensate for differences in growing season length between hemiboreal and Mediterranean climates, our turnover estimates fall within the range of Hagenbo *et al.,* (2017, 2018). However, our production estimates are generally higher than most previous estimates, suggesting a significant contribution of mycorrhizal mycelial production to belowground C fluxes in Mediterranean forest ecosystems. The overall fast production and turnover was evident from the fungal biomass reaching an apparent steady state around 2-3 months. Compared to boreal and temperate ecosystem forests, Mediterranean biomes are generally more P limited than N limited (Gill & Finzi, 2016), and a high N supply combined with low P availability have been shown to stimulate production of mycorrhizal mycelium under laboratory conditions (Wallander & Nylund, 1992). The stimulatory effect of P deficiency could be related to an increase in C supply as

carbohydrates pools in plants have been shown to increase under P limited conditions (Wallander & Nylund, 1992). Moreover, production of mycorrhizal mycelium is likely stoichiometrically constrained by availability of C and N, and the N demand of the host plant likely affects the amount of N available for assimilation and production of fungal biomass (Hagenbo *et al.*, 2019). A high mycelial production is likely possible when N relative to C is high (Schimel & Weintraub, 2003), and potentially a high N availability (relative to C and P) could contribute to the high mycelia production of the present study.

Production of mycorrhizal sporocarps was in total 143 kg ha^{-1} over the study period. Compared to the average mycelial production of 5.4 kg ha^{-1} day^{-1}, scaled up over the full length of the study period (230 days), production of mycorrhizal sporocarps represented 12% of the total mycelial production. This contribution of sporocarp growth is larger than estimates in Hagenbo *et al.,* (2019), where the growth of ectomycorrhizal sporocarps represented 0.4-7.3% of the mycelial production in *P. sylvestris* forest. Despite the relatively high sporocarps yield we did not observe any trade-off between sporocarps growth and mycelial biomass.

4.3 Methodological considerations

We were only able to quantify the average production and turnover rates over a July-February period, and the biomass declines observed in *Pinus* forests at early-autumn and early-winter - could either be related to a temporally decrease in production and/or an increase in turnover. However, since the observed seasonality in mycelial biomass ingrowth is similar to the bimodal seasonality of roots in Mediterranean forests (Alday *et al.*, 2020), it is possible that period of rapid root growth are also followed by periods of a high mycelial production. Although predicted and measured biomass was significantly correlated for all measurement time points, predictability of the biomass model (Eqn 1 with the production and turnover estimates) varied over the season, suggesting that our production and turnover estimates compare better to the natural production and turnover at certain time points of the season. For example, predictability was highest during October and December (R^2 = 0.78 and 0.53), intermediate for February (R^2 = 0.41), and lowest for September (R^2 = 0.30). Since the study was conducted between July-February, it is not surprising that predictability is greatest at the middle of the studied season.

Seasonal variation in production and turnover likely contributes to variation in predictability, and with the current approach we can only determine the average production and turnover rates over the study period. The biomass model in the present study is based on the assumption of stable production and turnover rates (Ekblad *et al.,* 2016), and violation of this assumption likely contributes to the variability of the production and turnover estimates, as observed in some of the study plots (plot 306, 312 and 320). Furthermore, we did not observe any significant differences in production and turnover over the different forest types, but it is possible that the number of forest plots per tree species was too low to obtain statistical support for forest type specific differences.

Another methodological consideration is the fact that we did not perform any DNA sequencing or stable isotope analyses to confirm that the fungal ingrowth of mesh bag was of mycorrhizal origin. In the study area, soil fungal biomass correlates with the abundance of mycorrhizal fungi which dominated the soil fungal community (53% of the total abundance), whereas free-living fungi (*e.g.* moulds yeasts, litter saprotrophs and pathogens) altogether accounts for 19% of the abundance, and taxa with unknown function represent 28% of the abundance (Castaño *et al.,* 2018b). Since sand-filled mesh bags have been demonstrated to select for mycorrhizal fungi over a wide different setting (Wallander *et al.*, 2001, 2010; Parrent & Vilgalys, 2007; Kjøller *et al.*, 2012), and based on the fact that mycorrhizal fungi dominates the soil fungal community and drive variation in soil fungal biomass (Castaño *et al.,* 2018b), it seems likely that our estimates are mainly represented by mycorrhizal fungi. Even though non-mycorrhizal fungi may enter the bag and even dominate the fungal community, in terms of relative abundance, they seem to not contribute to variation in biomass in mesh bags. For example, Hagenbo *et al.,* (2018) found that majority of the identified amplicon sequences was of non-mycorrhizal origin, in mesh bags incubated up to 97 days in hemiboreal forests. However, despite large relative abundance of non-mycorrhizal fungi, only amplicon number of mycorrhizal- and ericoid mycorrhizal fungi explained variation in biomass, suggesting that ruderal taxa and spores may enter the bags but contributes to biomass to a limited extent (Hagenbo *et al.,* 2018). Still, without community profiling and quantitative PCR we cannot rule out the possibility that non-mycorrhizal fungi contributed to the estimates to some extent, but likely their contributions are small.

Additionally, the mesh bags method is believed to select for fast-growing mycorrhizal species (Wallander *et al.*, 2013), potentially leading to overestimated production. While the mycelial production varies among mycorrhizal fungal species (Agerer, 2001), the mesh bags technique seems to be less biased in hemiboreal forests aged <60 years old (Hagenbo *et al.*, 2018). The extent of which missing species skew the biomass dynamics in mesh bags in Mediterranean forests is uncertain, but given the fact that most of the forest stands of the present study are aged about 60 years in age, it is possible that some sampling bias is involved in the production and turnover estimates presented here.

Sand as a growth substrate could also have biased the estimates to some extent as sand does not reflect the surrounding chemical and physical conditions of natural soil (Hendricks *et al*., 2006). Because sand lacks nutrients needed for growth it is possible that sand promotes resource re-allocation, and thus biomass turnover, to some extent. A potential way to decrease the importance of substrate choice is to minimise the size of the bags (Mikusinska *et al.*, 2013). We used mesh bags with a diameter of 2-cm which ensures that 75% of the bag volume is within 0.5 cm from the surface. Thus, given the dimension of the bag it is likely that that surrounding soil had a large influence on conditions inside the mesh bags, likely reducing the potential bias from using sand a growth substrate.

Finally, there are different model approaches to estimate mycelial dynamics from mycelial ingrowth mesh bags (Ekblad *et al.,* (2016), but based on the results of Hagenbo *et al.,* (2017) and (2018), obtained from the same study area but by using different approaches, it seems that the choice of method does not influence the estimate to a large extent.

4.4 Conclusions

We found that production and turnover rates of mycorrhizal fungal mycelium in Mediterranean forests is positively correlated with drought-moisture conditions, and we speculate that this is an effect of improved host tree performance when water restrictions are lifted. We observed that the seasonality in mycelial biomass in mesh bags was lower for *Q. ilex* forests than *Pinus spp.* forests, which may be explained by drought-resistant tree species are more capable of sustaining a stable mycorrhizal C supply. Overall, the results of this study highlight that restricted water access in Mediterranean ecosystem could be a limiting factor for mycorrhizal mycelial growth, and that

mycelial dynamics may shift under climate change, in response to decreased precipitation frequency, with consequences on tree performance and soil C cycling.

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6 Authors' contributions

A.H., C.C, S.d.M, J.G.A. and J.A.B designed and initiated the study. A.H., Y.P., C.C., J.G.A., and J.M.d.A conducted field work. A.H. and Y.P. processed the mesh bags and performed ergosterol assays. J.M.d.A processed and identified the sporocarps. J.M.d.A and J.A.B provided with environmental data. A.H. performed the statistical analyses and led the writing of the manuscript. All authors contributed critically to interpretations of results and to the drafts and gave final approval for publication.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Estimated mycorrhizal mycelial biomass production and turnover in relation to standardized precipitation index in Mediterranean forests dominated either by *Pinus pinaster*, *Pinus sylvestris* or *Quercus ilex*.

Table S1 Forest site characteristics.

Table S2 Mean sporocarp biomass during 2018 in Mediterranean forests dominated either by *Pinus pinaster*, *Pinus sylvestris* or *Quercus ilex*.

Table S3 Mycorrhizal mycelial biomass production and turnover estimates.

Table S4 Result of a multiple linear regression of incubation duration, dominant tree species and sampling time point in relation to the model error in Fig. 7a.

Table 1 Result of a multiple linear regression of incubation duration, dominant tree species and sampling time point in relation to variation in biomass of mycorrhizal mycelium in Mediterranean forests.

	Estimate	Std. Error	*T*-Value	*P*-Value	VIF
Intercept	170.9	50.2	3.40	**0.001**	
Incubation duration (days)	-0.6	0.7	-0.94	0.353	1.74
Dominant tree species					
Pinus pinaster	79.5	20.1	3.96	**<0.001**	1.22
Pinus sylvestris	-0.1	20.1	0.00	0.997	1.22
Quercus ilex	-79.4	21.6	-3.67	**0.001**	na
Harvest time					
05-Sept.	-50.3	31.0	-1.62	0.111	2.18
30-Oct.	52.5	24.7	2.12	**0.038**	1.95
18-Dec.	-70.0	32.9	-2.12	**0.038**	2.45
28-Feb.	67.7	29.0	2.34	**0.023**	na

Significant values ($P < 0.05$) are highlighted in bold. Adjusted $r^2 = 0.27$, n = 66.

Figure text

Figure 1 Incubation scheme of the mesh bags, showing intervals of incubation between July 2018 and February 2019. Beginning of arrows indicates installation time points of the mesh bags, and end of arrows indicates time points of harvests, *i.e.* early September, and late October, December and February. Number above arrows shows durations of incubations (days) and letters annotate mesh-bags with different incubation periods and indicates time points of installation. Each incubation period was represented by five mesh bags, and the full scheme was replicated across all eleven sites. The first two sets of mesh bags (a and c) were installed at the 11th of July 2018 and the two final sets of mesh bags (b and f) were harvested at the 28th of February 2019.

Figure 2 Seasonal variations in mycorrhizal mycelial biomass in ingrowth mesh bags incubated in Mediterranean forests dominated by (a) *Pinus pinaster*, (b) *Pinus sylvestris* and (c) *Quercus ilex*. Red and blue bars in (a-c) represents biomass estimates derived from mesh bags incubated over two and

four months, respectively. Correspondingly, red and blue horizontal dashed lines in (a-c) represent mean biomass of mesh bags incubated over two and four months. Parentheses in the axis labels of a-c indicates the incubation durations of the mesh bags. Solid lines in (d) represents a loess (locally estimated scatterplot smoothing) regression fitted to biomass estimates from mesh bags incubated for two months in *Pinus pinaster* (red), *Pinus sylvestris* (green) and *Quercus ilex* (blue) forests. The grey area in (d) represents the 95% confidence interval for loess regression fitted to the *Q. ilex* and *Pinus* spp. data. Lower and upper whiskers represent the first and third quartile multiplied by 1.5, respectively. For summary statistics see table 1.

Figure 3 Monthly mean sporocarp production of mycorrhizal (blue bars) and non-mycorrhizal fungi (red bars) in Mediterranean forests dominated by trees of *Pinus pinaster* (a), *Pinus sylvestris* (b) and *Quercus ilex* (c). Percentages indicate the relative proportion of mycorrhizal fungal sporocarps data, derived from the year of 2018.

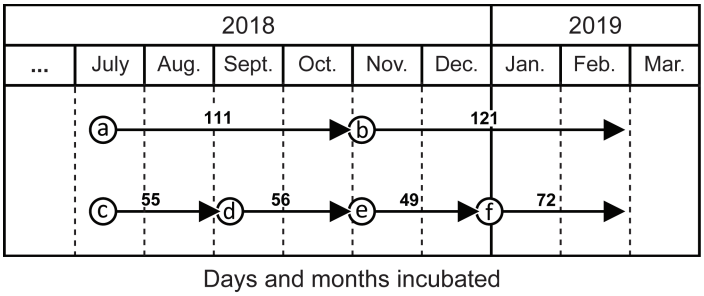
Figure 4 Estimated biomass production (a) and turnover (b) of mycorrhizal mycelium in Mediterranean *Pinus pinaster* (red), *Pinus sylvestris* (green) and *Quercus ilex* (blue) forests. Balloons represents the kernel density distribution interval for the production and turnover estimates when using parametric bootstrapping to repeatedly fit Eqn 1 to biomass values which have been resampled 500 times, based on mean and standard deviation of the technical replicates of biomass (n = 3-6). Open circles represent the mode values of the parametric estimates and dashed lines represent the means of the mode values. Outliers are indicated by crosses and represents data point with values smaller than the first quartile, multiplied by 1.5, or greater than the third quartile, multiplied by 1.5. Numbers indicate different forest sites.

Figure 5 Variation in mycorrhizal mycelial biomass production (a) and turnover (b) in Mediterranean forests dominated either by *Pinus pinaster*, *Pinus sylvestris* or *Quercus ilex*, and over all forest types combined. Whiskers represent the lower and upper interquartile range multiplied by 1.5. One outlier in (b) represented by a *Quercus ilex* forests with a turnover of 66.3 times $year^{-1}$ is excluded from the

figure and omitted in the calculations of median and quartile ranges. Production and turnover estimates represent mode values from parametric bootstrapping (Fig. 4).

Figure 6 Estimated mycorrhizal mycelial biomass production (a) and turnover (b) in relation 3-month standardized precipitation index (SPI) in Mediterranean forests dominated either by *Pinus pinaster* (red circles), *Pinus sylvestris* (green circles) or *Quercus ilex* (blue circles). Values of SPI represent the average SPI over the study period July-February. Production and turnover estimates represent mode values from parametric bootstrapping (Fig. 4) and lines represent linear regression models fitted to the data with *P*- and R^2 values from the model fits shown in the lower right corners of the plots. A negative SPI indicates low water availability and values between 0 to -0.99 indicates mild drought conditions relative to previous years (McKee *et al*. 1993). The shaded grey areas indicate limits of the 95% confidence interval of the regressions. See Supporting Information Fig. S1 for correlations with 1- and 2-month SPI. Functions of regression models: $y_a = 36.0+ 82.2x$; $y_b = 171 + 422x$.

Figure 7 Predicted mycorrhizal mycelial biomass compared against the measured biomass in mycelial ingrowth bags incubated in Mediterranean forests dominated by trees of *Pinus pinaster* (red circles), *Pinus sylvestris* (green circles), and *Quercus ilex* (blue circles). Predictions are calculated from Eqn 1, using the production and turnover estimates in Figure 3-4, and all biomass estimates represent mean values derived from five ingrowth bags. The different comparisons (a-e) are based on biomass data collected over (a) the entire study period and in (b) September, (c) October, (d) December and (e) February. Lines represent linear regression models fitted to data and *P*- and R^2 values from the model fits are shown in the lower right corners of the plots. Shaded grey indicated limits of the 95% confidence interval of the regressions.



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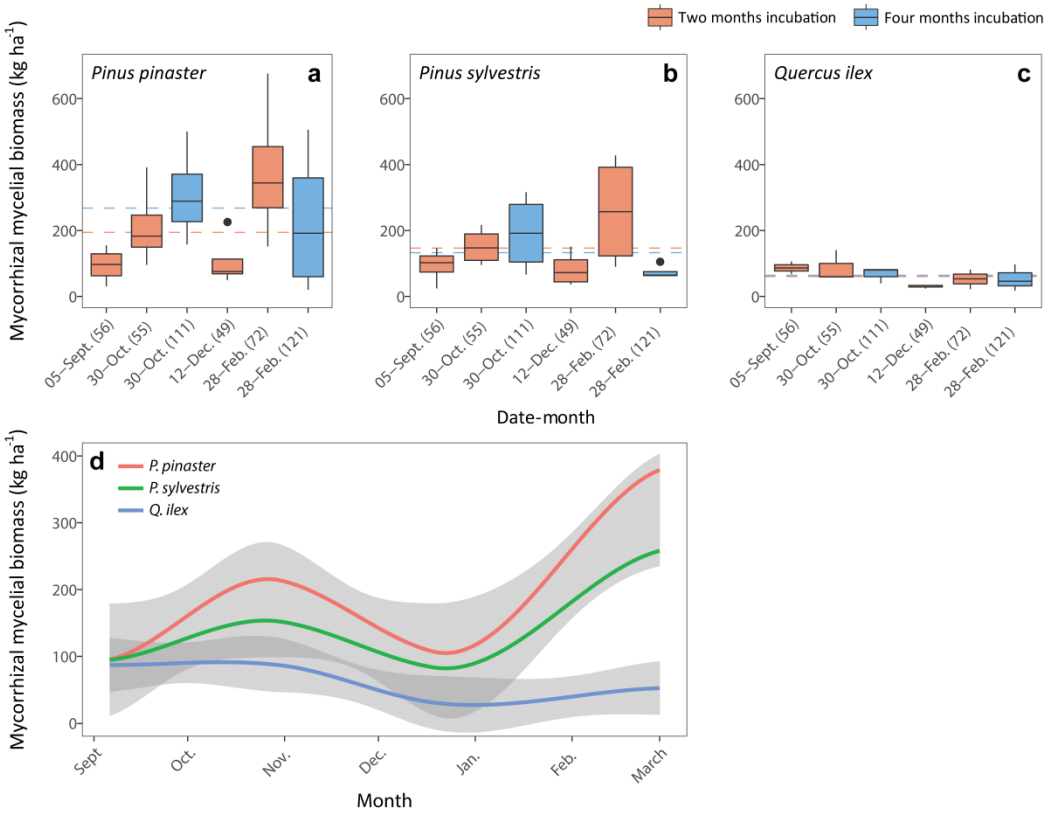

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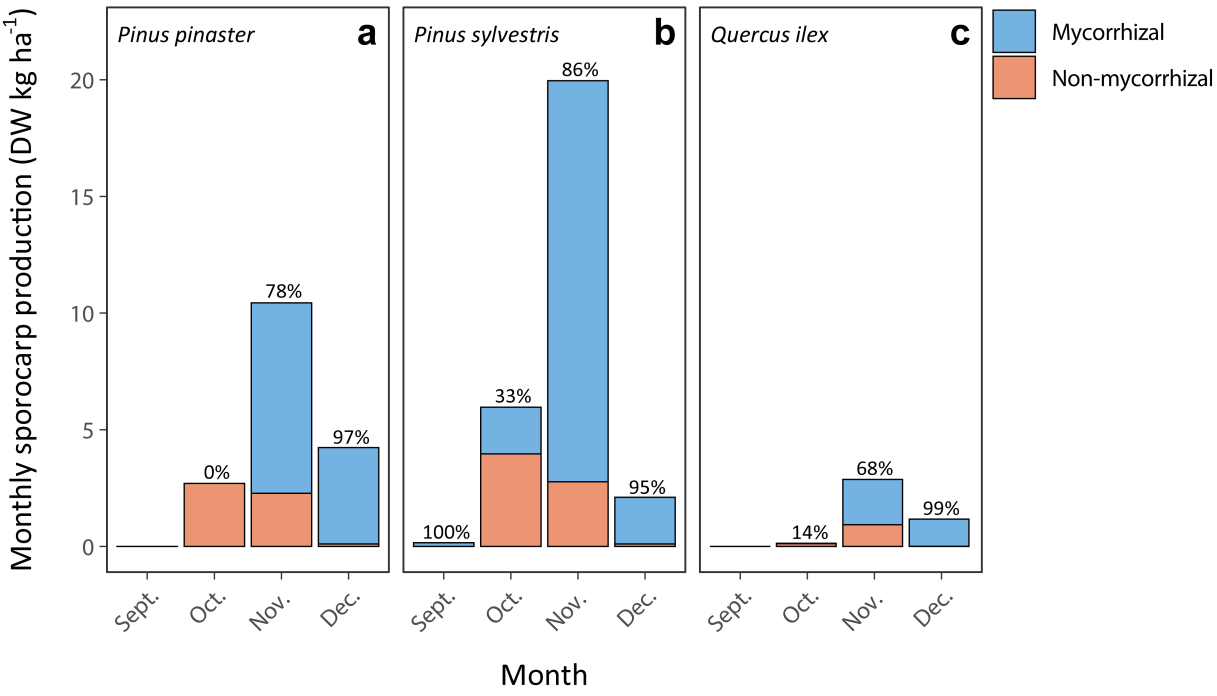

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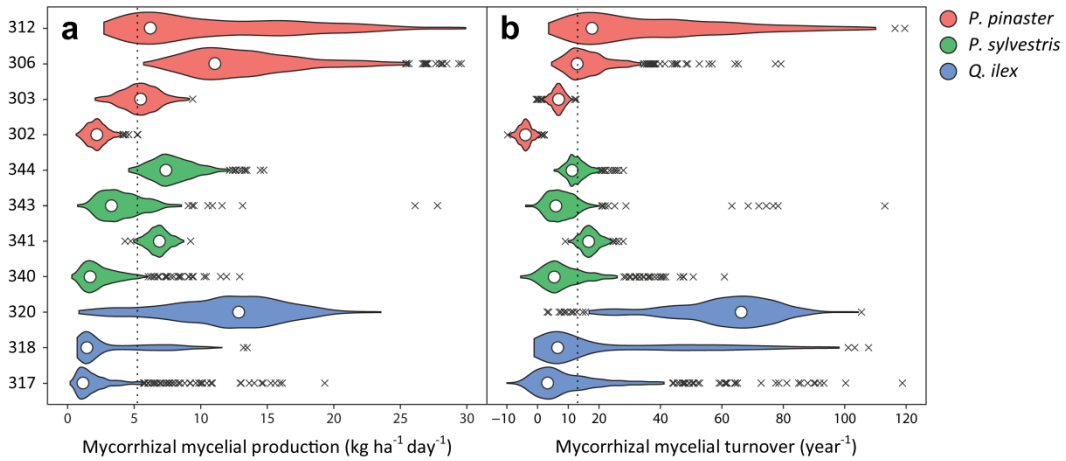

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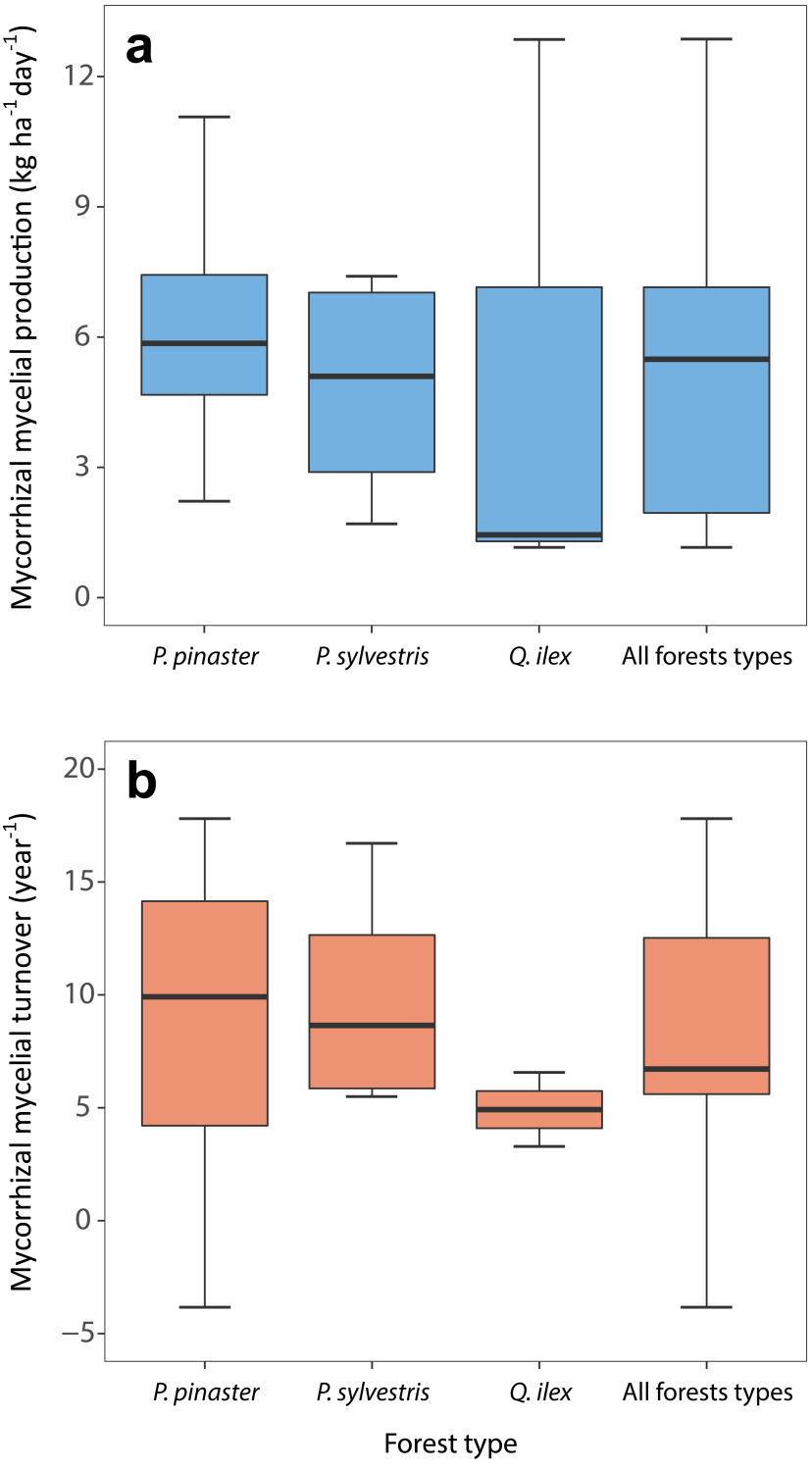

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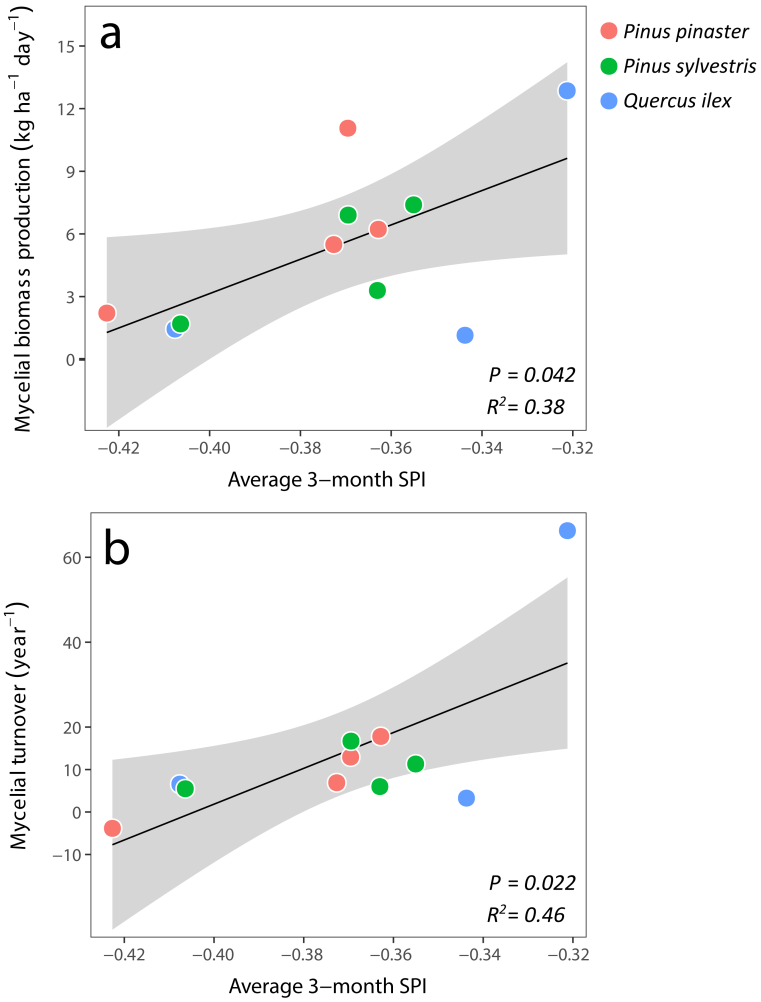

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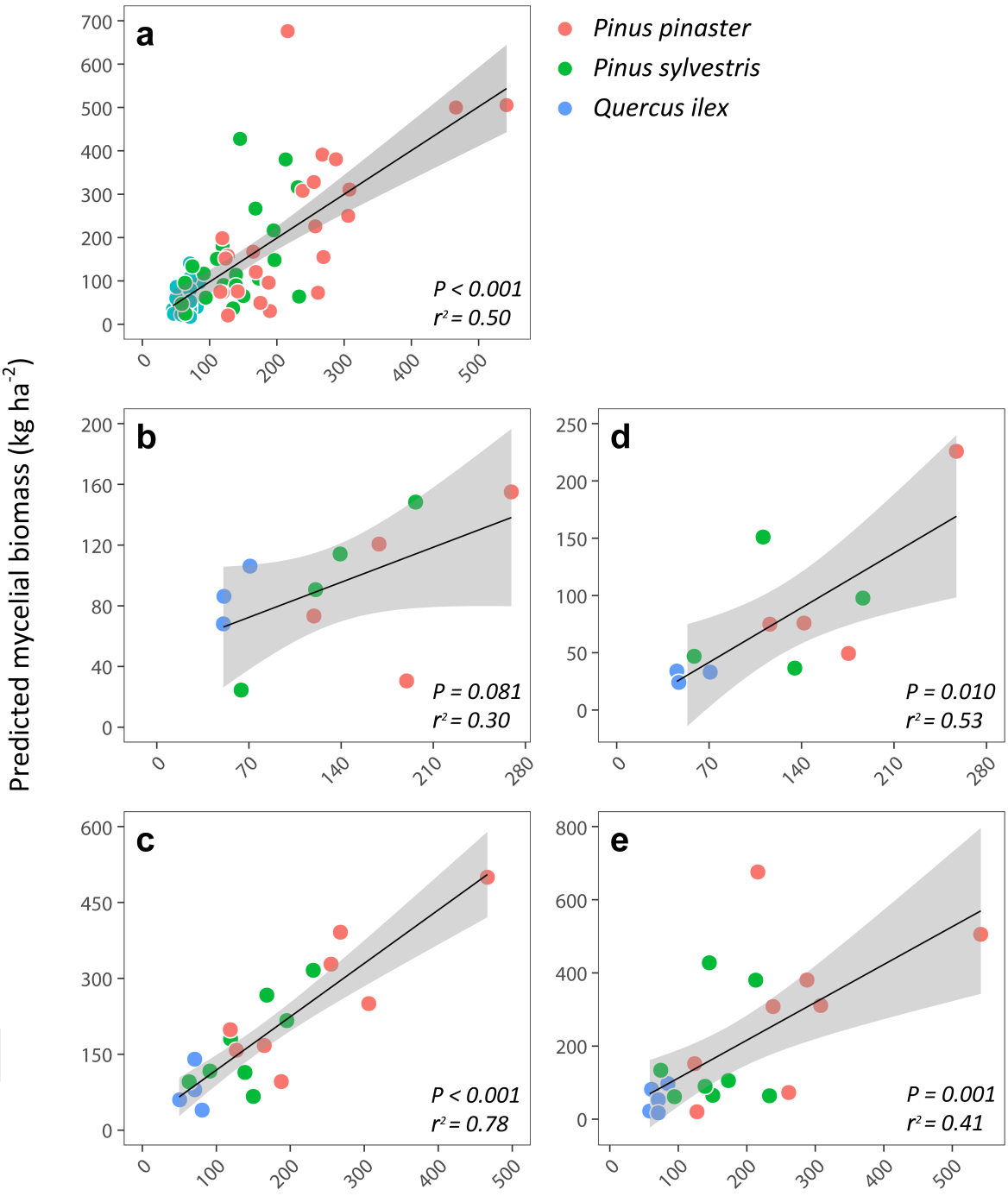

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